METHOD FOR AMPLIFICATION OF RNA SEQUENCES

The present invention relates to a method for amplification of target RNA sequences, to primers for the amplification of target RNA sequences and to a kit comprising one or more of the primers.

Nucleic acid amplification methods, wherein target RNA or DNA sequences are amplified, are generally applied in the field of molecular biology, biochemistry and biotechnology and the possible applications are numerous and still increasing. The amplification methods increase the number of copies of a particular target nucleic acid sequence, which often is present in small amounts and in an environment in which a wide variety of other nucleic acids, both RNA and DNA, are present.

Nucleic acid amplification methods are in particular used to facilitate the detection and quantification of specific target nucleic acid sequences present in samples such as blood, sperm, tissue, hair, microorganisms, cells, tumors etc., in diagnosing, for example, infectious diseases, inherited diseases, and various types of cancer. In addition, nucleic acid amplification methods have found their applications in other fields where samples are investigated in which informative target nucleic acids may be present in minute amounts, such as in forensic sciences and in archeology or to establish paternity.

Several nucleic acid amplification techniques are known, like Polymerase Chain Reaction (PCR) and Transcription Based Amplification (TBA), which are based on different mechanisms of action, but all depend on the annealing of one or more primers to the boundaries of the target nucleic acid sequence or its complementary sequence. The annealing of a primer to the boundary of the target nucleic acid sequence is provided by the hybridization of a contiguous number of

nucleotides comprised in the primer, to a contiguous number of complementary nucleotides in the target nucleic acid sequence.

Efficient hybridization of the primer generally requires at least 15 contiguous complementary nucleotides. If less nucleotides are present, the ratio between specific and non-specific annealing of the primer decreases below a point where no longer effective amplification of the target nucleic acid can occur. The requirement of at least 15 contiguous complementary nucleotides, however, limits the possibility of for example amplification of target nucleic acids in case only a consensus sequence of the target sequence can be used, especially in cases when all genotypes from target nucleic acids derived from organisms like viruses, bacteria, and yeast, or from tissue specimens like blood, cells, tumors, and lymphoid fluids are to be amplified. When, due to genetic variation, the consensus sequence may vary among individual species, inefficient hybridization of the primers as a consequence of the presence of non-complementary nucleotides may occur.

If several "genetic variations" of a target nucleotide sequence are known, conserved stretches of nucleotides can be identified and used for primer design. However, preferred conserved stretches in some species, like RNA viruses, such as parts of the coding region of the human hepatitis C virus, are sometimes too small to be used to design efficient primers. In addition, even these conserved segments have a tendency to mutate, which is most pronounced in fast dividing organisms like viruses and bacteria, resulting in a reduced efficiency of hybridization of the primer. As a consequence, amplification may not occur, which is highly undesirable in for example routine diagnostics

since a false negative amplification result can have severe consequences for the patient concerned.

Therefore there is a clear need for an amplification method which allows for amplification using small stretches of target RNA sequences and which method is less prone to mutations or genetic variations in the hybridizing segment of the target sequence.

In the research that led to the present invention it has been found that the above-mentioned problems can be obviated by an amplification method comprising the steps of:

(a) annealing a first primer to the target RNA sequence, said first primer comprising a hybridizing sequence, which is complementary to and hybridizes to at least a first segment of the target RNA sequence, operatively associated with a promoter sequence;

(b) extending said first primer in a reaction catalyzed by a DNA polymerase, forming a first RNA/cDNA hybrid nucleic acid molecule;

(c) selectively removing the target RNA sequence of the first RNA/cDNA hybrid nucleic acid molecule forming a first single stranded cDNA sequence;

(d) annealing a second primer to the obtained first single stranded cDNA sequence, said second primer comprising a hybridizing sequence which is complementary to and hybridizes to a first segment of the first single stranded cDNA sequence;

(e) extending said second primer in a reaction catalyzed by a DNA polymerase to form a first double stranded DNA molecule; and

(f) employing the first double stranded DNA molecule of step (e) in the preparation of a plurality of RNA transcripts that are complementary to the target RNA sequence in a reaction catalyzed by a DNA-dependent

RNA polymerase with specificity for the promoter sequence comprised in the first primer;

wherein the first primer comprises a hybridizing sequence of 7 to 14 nucleotides, a transcription enhancing sequence, and an anchor which is capable of binding to a second segment of the target RNA sequence, and/or wherein the second primer comprises a hybridizing sequence of 7 to 14 nucleotides, an amplification enhancing sequence and an anchor which is capable of binding to a second segment of the first single stranded cDNA.

According to the present invention it has surprisingly been found that a reduction of the number of contiguous hybridizing nucleotides in at least one of the primers still allows for a reliable amplification method if said primer additionally comprises an anchor which binds to a second segment of the target RNA sequence, which is not the hybridizing nucleotide sequence, in such a way that the anchor exposes the hybridising sequence to the first segment.

The use of an anchor in addition to a hybridizing sequence thus results in a two-step annealing process: 1) binding of the anchor to a segment of the target RNA sequence, designated as the second segment, and 2) hybridization of the hybridizing sequence of the primer to the contiguous complementary nucleotides of the target RNA sequence, designated as the first segment, which steps can occur simultaneously or sequentially (i.e. first step 1 and then step 2 or first step 2 and then step 1). The anchor may bind specifically, but the anchor and hybridizing sequence may also together be responsible for the specific interaction of the primer to the target RNA, for example in case the anchor is 7-14 nucleotides in length and the hybridizing sequence is 13-7 nucleotides in length, by which the anchor

and hybridizing sequence independently do not bind specifically, but the combination is specific.

According to the invention, the number of nucleotides in the hybridizing sequence of the primers can be reduced to 14, 13, 12, 11, 10, 9, 8, or 7 nucleotides. Due to the presence of the anchor in the primer, specific binding of the primer to the target nucleotide sequence will occur, regardless of the fact that the hybridizing part only contains maximally 14 nucleotides, and in itself would not be able to hybridize specifically. Thus, by the combination of the anchor and the hybridizing sequence specific binding of the primer to the target sequence is accomplished.

The method according to the invention is further explained referring to Figure 16, wherein a preferred embodiment of the method is shown. During the first step the PNA anchor present in the first primer (P1), usually designated as the forward primer, binds to a segment of the target RNA sequence (RNA+). Subsequently, the hybridizing sequence (H) of the first primer hybridizes to the first segment of the target RNA sequence.

After annealing of the first primer to the target RNA sequence, the primer is extended using a DNA polymerase, such as the avian myeloblastosis virus (AMV) reverse transcriptase polymerase. The DNA polymerase will copy the target RNA sequence forming a first RNA/cDNA hybrid molecule.

Simultaneously, or subsequently the target RNA sequence is selectively removed, preferably by enzymatic digestion with for example an RNase. The resulting first single stranded cDNA sequence (DNA-) is used as a template for the subsequent step in the amplification process.

This subsequent step requires the annealing of a second primer (P2), usually designated the reverse primer, to the formed first single stranded cDNA. According to the shown

embodiment, the second primer also comprises a hybridizing sequence (H) which is complementary to and hybridizes to a first segment of the single stranded cDNA, an amplification enhancing sequence (X) and a PNA anchor which is capable of specific binding to a second segment of the first single stranded cDNA sequence.

According to the present invention, both the first and second primer, or only the first or second primer, may comprise a short hybridizing sequence and an anchor.

In a subsequent step of the amplification method, the second primer is extended by the AMV reverse transcriptase polymerase using the first single stranded cDNA sequence as a template resulting in a first double stranded DNA molecule including a double stranded promotor sequence derived from the first primer.

This first double stranded DNA molecule is subsequently used for the preparation of a plurality of RNA transcripts (RNA-) by a DNA-dependent RNA polymerase. The sequence downstream of the promotor site is used as template meaning that the RNA transcripts will not include the anchor sequence of the forward primer.

It will be obvious for the person skilled in the art that the choice of the DNA-dependent RNA polymerase is dependent on the choice of the promoter sequence in the first primer. In a preferred embodiment of the present invention, the promoter sequence is the T7 promoter sequence and the DNA-dependent RNA polymerase is the T7 polymerase.

In a further preferred embodiment, the method according to the invention further comprises the following steps:

(g) annealing of the second primer to the RNA transcripts produced in step (f);

(h) extending the second primer in a reaction catalyzed by the DNA polymerase to form a second RNA/cDNA hybrid nucleic acid molecule;

(i) selectively removing the RNA of the second RNA/cDNA hybrid molecule to obtain a second single stranded cDNA molecule;

(j) annealing the first primer to the obtained second single stranded cDNA sequence;

(k) extending the 3' end of the second single stranded cDNA molecule in a reaction catalyzed by the DNA polymerase using the first primer as a template to form a second partly double stranded DNA molecule comprising a double stranded promotor site;

(l) employing the second partly double stranded DNA molecule of step (k) in the preparation of a plurality of RNA transcripts complementary to the target RNA sequence in a reaction catalyzed by the DNA-dependent RNA polymerase with specificity for the promotor sequence in the first primer.

These additional steps of the amplification method according to the present invention result in a self-sustained (or cyclic) amplification of the target RNA sequence, which is also schematically represented in Figure 16B.

In a preferred embodiment of the present invention, the first primer comprises, going from the 5' end to the 3' end, an anchor, a transcription enhancing sequence, and a hybridizing sequence consisting of 7 to 14 nucleotides which are complementary to a first segment of the target RNA sequence of 7 to 14 contiguous nucleotides.

In a further preferred embodiment, the second primer comprises of, going from the 5' end to the 3' end, an anchor, an amplification enhancing sequence, and a hybridizing sequence consisting of 7 to 14 nucleotides which are

complementary to a the first segment of the first single stranded cDNA sequence of 7 to 14 contiguous nucleotides.

Preferably, the hybridizing sequence of the first and second primer comprises 7 to 10 nucleotides which are complementary to a the first segment of the RNA target sequence or the first single stranded cDNA sequence of 7 to 10 contiguous nucleotides, respectively.

Due to the presence of the anchor, the primer is able to bind specifically to the target RNA sequence. The hybridizing sequence alone would not be able to bind specifically, due to its short length.

According to the invention, the anchor can be any compound which is capable of binding to a second segment of the target RNA sequence. The anchor preferably is an, optionally modified, oligonucleotide or a protein. Preferred is an, optionally modified, oligonucleotide which comprises 7-22, optionally modified, nucleotides, more preferably 7-14, most preferably 9-14 nucleotides, whichs bind to the second segment of the target RNA sequence or the second segment of the single stranded cDNA sequence. The person skilled in the art will understand that for binding of the anchor it is not required that all nucleotides in the anchor are complementary to the corresponding sequence in the second segment of the target RNA sequence. Thus, for specific binding of the primer to the target RNA sequence approximately at least 90% of the nucleotides of the anchor should be complementary. This allows for a more flexible selection of the second segment without influencing the efficiency of the amplification process.

The nucleotides of the anchor can be both RNA and DNA, or modified nucleotides, such as locked nucleic acids (LNA) or 2'-O-methyl modified nucleotides, or peptide nucleic acids (PNA). Such modifications provide either a more stable

anchor or enhance the specific binding characteristics of the primer.

Proteins, or fragments derived thereof, which display specific binding to nucleic acid sequences can also be used as the anchor. Proteins, or fragments derived thereof, which are capable of binding to the second segment of the target RNA sequence or the second segment of the first single stranded cDNA sequence can be selected or designed by for example protein engineering. Examples of such proteins are polyC-binding proteins, polyA-binding proteins, proteins comprising one or more zinc-finger, restriction enzymes, and/or antibodies and fragments thereof such as scFvs, Fabs etc.

The first and second segment of the target nucleic acid sequence preferably are separated by 0, 1, 2, 3, 4, 5 or 6 nucleotides, more preferably by 0, 1, 2, 3 or 4 nucleotides, and most preferably by 0, 1, 2 or 3 nucleotides. When more than 6 nucleotides are present between the two segments, the efficiency of the two-step annealing of the primer and thus of the amplification is reduced.

The single stranded RNA transcripts produced during amplification are extremely suitable for the use of hybridization-based detection systems with sequence-specific probes. The detection may take place at the end of the amplification using electro-chemiluminescence (ECL). A specific capture probe, attached to magnetic beads via streptavidin-biotin interaction, is used to immobilize the RNA transcript and a ruthenium-labeled detection probe hybridizes with the transcript. Also other currently known detecion methods may be used, such as for example "enzyme linked gel assay" (ELGA) or fluorescence spectroscopy.

A very elegant detection method is the use of molecular beacons. Molecular beacons are DNA oligonucleotides

labeled with a fluorophore at the 5'-end. The last part of the 3'-end of the sequence is complementary to the first part of the 5'-end and a hairpin stem is formed in such as way that a quencher at the 3'-end absorbs the emitted light of the fluorophore. The hairpin loop sequence is complementary to the target sequence of the RNA transcript. Because of the binding of the loop sequence to the target sequence, the hairpin stem opens up and the quencher becomes separated from the fluorophore. The increase in light emitted can be detected by a fluorometer. Hybridization with the RNA transcript takes place during amplification enabling "real-time" detection. To quantify the amount of target nucleic acid in a sample, a calibrator may be included, which is added prior to the isolation of the nucleic acid and is co-extracted and co-amplified with the target nucleic acid.

The detection probe may be a target-specific probe or may be a generic probe, i.e. directed against a generic sequence incorporated in the RNA transcript, such as a sequence identical to the amplification enhancing sequence.

Thus, in a preferred embodiment of the method according to the invention, the target-specific probe hybridizes to the RNA transcript, complementary to the target RNA sequence. According to another preferred embodiment, the generic probe hybridizes to the sequence identical to the amplification sequence of the second primer, enabling the use of one sequence-specific probe for the detection of a wide variety of different amplified target RNA sequences. This results in a cost effective detection method (detection probes are in general relatively expensive to synthesize), and increases the chances for reproducible and quantitative detection, since the binding characteristics of the probe could be comparable in every amplification method regardless of the starting materials or the target RNA sequence.

The amplification method of the present invention is preferably used for the amplification of viral target RNA sequences, such as RNA isolated from the human immunodeficiency virus (HIV) or RNA isolated from the human hepatitis C virus, which viruses have only partially conserved nucleic acid sequences.

The invention further relates to primers comprising an anchor and a hybridizing sequence. The primers according to the invention preferably comprise, going from the 5' end to the 3' end, an anchor, as defined above, a transcription enhancing sequence or an amplification enhancing sequence, as defined below, and a hybridizing sequence of 7 to 14 nucleotides, preferably 7 to 10 nucleotides, as defined above.

The use of the primers comprising an anchor and a hybridizing sequence is not limited to Transcription Based Amplification (TBA), but these primers can also be used in other amplification methods like the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

According to another aspect of the present invention, the invention relates to a kit for the amplification and/or detection of a target RNA sequence comprising at least one or more primers according to the invention.

The kit according to the invention may further comprise one or more sequence-specific probes, an amplification buffer, and/or one or more enzymes, such as a DNA polymerase, RNAse H, and a DNA-dependent RNA polymerase.

DEFINITIONS

According to the present invention a target RNA sequence is defined as a specific segment of RNA which is to be amplified by an amplification method.

Hybridization is defined as the binding of a contiguous number of nucleotides, present in for example a primer, to the complementary nucleotides of for example a target RNA sequence. The hybridization results in the formation of a double stranded nucleic acid molecule.

Annealing is defined as the association of a primer with a target nucleic acid sequence by for example hybridization.

Specific binding is defined as the preference for binding of a compound (such as a primer, or part of a primer) to a specific oligonucleotide sequence, as compared to the binding of said compound to a random nucleotide sequence under the used conditions. Thus a compound binds specifically if the compound only binds to one and the same nucleotide sequence.

A nucleic acid sequence is designated complementary if all individual nucleotides of the nucleic acid sequence are complementary, for example, a oligonucleotide sequence reads 5'-ATG ACC TGG-3' and the complementary oligonucleotide sequence reads 3'-TAC TGG ACC-5'.

A template is defined as a nucleic acid molecule that is to be transcribed by a polymerase. The synthesized transcript is complementary to the nucleic acid molecule, or at least to one strand of the nucleic acid molecule. Both RNA and DNA are usually synthesized in the 5' to 3' direction.

A transcription enhancing sequence is a nucleic acid sequence comprising a promotor, for example T7, which is a non-specific sequence vis-à-vis the target sequence. The transcription enhancing sequence may optionally comprise a purine stretch (mainly composed by G and/or A). It creates a loop between the anchor and the hybridizing sequence.

An amplification enhancing sequence is a non-specific nucleic acid vis-à-vis the target sequence, but it comprises

no promotor, i.e. only a random sequence that generates a loop between the anchor and the hybridizing sequence.

The invention will be further exemplified by the following Figures and Examples. These Figures and Examples are not intended to limit the present invention in any way.

FIGURES

Figure 1 shows the legend used in the graphs of the subsequent figures.

Figure 2: Amplification of HIV RNA (RNA transcript, 500, 50 and 5 cps) with an anchor p1 primer (Fig. 2B: HIV12, table 1) or a standard p1 primer (Fig. 2A: HIV1, table 1), both in combination with a standard p2 primer (HIV2, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 3: Schematic representation of anchor p1 primer HIV12 (table 1) and primer HIV11 (table 1) without anchor.

Figure 4: Amplification of HIV RNA (RNA transcript, 500, 50 and 5 cps) with a standard p1 primer (Fig. 4A: HIV1, table 1), an anchor p1 primer (Fig. 4C: HIV12, table 1), a p1 primer without anchor (Fig. 4B: HIV11, table 1) or a combination of primer HIV12 and HIV11 (Fig. 4D). All in combination with a standard p2 primer (HIV2, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 5: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with a standard p1 primer (Fig. 5A: HIV1, table 1), anchor primers with different anchor lengths varying from 22 to 7 nucleotides (HIV12 (Fig. 5B), 17 (Fig. 5C), 20 (Fig. 5D), 21 (Fig. 5E), 22 (Fig. 5F), table 1). All in combination with a standard p2 primer (HIV2, table 1) and

a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 6-8: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with anchor primers with different anchor lengths varying from 14 to 9 nucleotides (HIV17 (Fig. 6A), 20 (Fig. 7A), 21 (Fig. 8A), table 1) and the same primers containing anchors with 2'-O-Methyl nucleotides (HIV17 MET (Fig. 6B), HIV20 METa & b (Fig. 7B and C), HIV21 METa & b (Fig. 8B and C), table 1). All in combination with a standard p2 primer (HIV2) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 9: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with a standard p1 primer (HIV1, table 1), anchor primers (HIV17 and HIV22, table 1) and p1 primers containing anchors with LNA nucleotides (HIV22 LNA1-4, table 1). All in combination with a standard p2 primer (HIV2, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control. Fig. 9A: standard P1 primer HIV1; Fig. 9B: anchor primer HIV22 LNA1; Fig. 9C: anchor primer HIV22 LNA2; Fig. 9D: anchor primer HIV17; Fig. 9E: anchor primer HIV22; Fig. 9F: anchor primer HIV22 LNA3; Fig. 9G: anchor primer HIV22 LNA4.

Figure 10: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with anchor primers (Fig. 10A: HIV17 and Fig. 10C: HIV21, table 1) and p1 primers containing PNA anchors (Fig. 10B: HIV17 PNA and Fig. 10D: HIV21 PNA, table 1). All in combination with a standard p2 primer (HIV2, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 11: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with standard p2 primers (HIV2 (Fig. 11A) and HIV26 (Fig. 11B), table 1) and anchor p2 primers (HIV27

(Fig. 11C) and HIV29 (Fig. 11D), table 1). All in combination with a standard p1 primer (HIV1, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 12: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with anchor p2 primers with different anchor lengths varying from 22 to 14 nucleotides (HIV27 (Fig. 12A), 31 (Fig. 12C), 32 (Fig. 12D), 33 (Fig. 12E), 29 (Fig. 12B), table 1). All in combination with a standard p1 primer (HIV1, table 1) and a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 13: Amplification of HIV RNA (Total viral RNA, 500, 50 and 5 cps) with an anchor p1 primer (HIV17, table 1) in combination with a standard p2 primer (HIV2, table 1) (Fig. 13B or anchor p2 primers (HIV27 (Fig. 13C) and HIV29 (Fig. 13D), table 1). The standard primer set (HIV1/ HIV2, table 1) is used as a reference (Fig. 13A). All in combination with a molecular beacon (HIV-MB-WT, table 1) as probe. A sample without template (NT) is used as negative control.

Figure 14: Amplification of HCV RNA (RNA transcript, 5×10^5, 5×10^4 and 5×10^3 cps) with an anchor p1 primer (HCV13 (Fig. 14B) or HCV 46 (Fig. 14C), table 2) or a standard p1 primer (HCV1 (Fig. 14A), table 2), both in combination with a standard p2 primer (HCV2, table 2) and a molecular beacon (HCV-WT3, table 2) as probe. A sample without template (NT) is used as negative control.

Figure 15: Amplification of HCV RNA (RNA transcript, 5×10^6 and 5×10^5 cps) with an anchor p1 primer (HCV22 (Fig. 15B), table 2), having a 3 nt gap between the anchor sequence and the 3' hybridizing sequence. Anchor p1 primer HCV13 (Fig. 14A)(table 2) is used as reference. Both anchored

primers are combined with the standard p2 primer (HCV2, table 2) and molecular beacon (HCV-WT3, table 2). A sample without template (NT) is used as negative control.

Figure 16: Scheme showing a preferred embodiment of the method according to the invention. P1: first primer; Y: transcription enhancing sequence; T7: T7 promotor sequence, H: hybridizing sequence; P2: second primer; X: amplification enhancing sequence; H: hybridizing sequence.

EXAMPLES

EXAMPLE 1

Amplification of HIV RNA with anchor p1 primer

Scott Layne HIV particles (HIV-infected cells (Layne et al (1992), Virology **189**: 695-714) lysed with lysisbuffer (NucliSens, BioMérieux)) or HIV gag RNA transcript were used as input material for amplification. Inputs of 500, 50 and 5 cps were tested. Amplification was performed in NASBA buffer (40 mM Tris-HCl pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 15% v/v DMSO, 5 mM DTT, 1 mM each dNTP, 2 mM ATP, 2 mM CTP, 2 mM UTP, 1.5 mM GTP, 0.5 mM ITP, 0.2 µM of each primer (forward primer (P1) and reverse primer (P2), table 1), 0.1 µM molecular beacon probe (HIV-MB-WT, table 1). The mixture was incubated for 2 min at 65 °C to denature the RNA and for 2 min at 41°C, to hybridize the P1 primer to the target. Subsequently, NASBA enzymes (0.08 units RNase H, 32 units T7 RNA polymerase, 6.4 units AMV reverse transcriptase and 2.1 µg BSA) were added, the reaction mixture was mixed by gently tapping and short centrifugation, and the amplification and real-time detection was started. The reaction mixture was incubated at 41°C in the NucliSens EasyQ Analyzer (NucliSens, BioMérieux) for 60 minutes with fluorescence monitoring every minute. The

reactions were excited at 485 nm and the emission signal was measured at 518 nm.

Amplification with an anchor p1 primer (HIV12, table 1) was performed in combination with a standard p2 primer (HIV2, table 1). The anchor primers consist of a 22nt anchor sequence upstream of the T7-promoter sequence and 7nt hybridizing sequence downstream of the T7-promoter sequence. Amplification with standard p1 primer (HIV1, table 1) and standard p2 primer (HIV2, table 1) was used as reference. The results show that sensitivity of amplification with the anchor primer is comparable to the reference primer (figure 2).

EXAMPLE 2

Amplification of HIV RNA with p1 primer with very short hybridisation sequence

Amplification with a p1 primer identical to HIV12, but no anchor sequence (HIV11, table 1, figure 3), containing only the 3′ hybridizing target specific sequence of 7 nucleotides, was performed in combination with a standard p2 primer (HIV2, table 1). HIV1 and HIV12 (table 1) were used as reference. Amplification was performed as described in example 1. As shown in figure 4, no amplification was observed with primer HIV11 (Fig. 4B), indicating that the anchor sequence is required for efficient amplification. The anchor is assumed to be necessary during the initiation of the reaction and not in the cyclic phase. Therefore, addition of a non-anchored primer can be of advantage during the cyclic phase of the reaction. When using both the anchor primer HIV12 and primer HIV11 in one and the same amplification reaction, a slight improvement of the reaction kinetics was observed (figure 4D).

EXAMPLE 3

Amplification of HIV RNA with anchor p1 primers with different anchor lengths

To investigate what minimum length of the anchor, still results in a specific amplification, primers with different anchor lengths were designed. Amplification with anchor primers with different anchor lengths (deleted from the 5'end) varying from 22 to 7 nucleotides (HIV12, 15, 16, 17, 20, 21, 22, table 1) was performed in combination with a standard p2 primer (HIV2, table 1). The standard p1 primer HIV1 (table 1) was used as reference. Amplification was performed as described in example 1. Deletion up to 14 nucleotides still results in an efficient amplification as compared to the references (HIV1 standard primer and HIV12 anchor primer) (figure 5). However, anchor lengths below 14 nucleotides show very little or no amplification. The hybridization temperature of 14 nucleotides or more is expected to be higher then 41°C (amplification temperature). Shorter anchors could have hybridization temperatures below 41°C, which may explain inefficient amplification.

EXAMPLE 4

Amplification of HIV RNA with anchor p1 primers using 2'-O-Methyl modified nucleotides in anchor sequence

In order to increase the binding efficiency of the anchor, 2'-O-Methyl modified nucleotides are incorporated in the anchor. Primers with anchor lengths of 14nt (HIV17 MET, table 1), 12nt (HIV20 METa & b, table 1) , 9nt (HIV21 METa & b, table 1) and 7nt (HIV22 METa & b) were used in amplification. The expected melting temperature increase is about 1.5°C per incorporated 2'-O-Methyl modified nucleotide.

A standard p2 primer (HIV2, table 1) was used as reverse primer. Amplification was performed as described in example 1. Using these anchor primers with 2'-O-Methyl modified nucleotides clearly improves the sensitivity of the amplification reaction (figures 6-8), in particular when using anchors shorter then 14 nucleotides. (12 & 9nt anchors HIV20 MET and HIV21 MET). 7nt anchor primers with 2'-O-Methyl modified nucleotides (HIV22 MET) turn out to be too short (result not shown). Probably, 7 nucleotide anchors, even with 2'-O-Methyl modified nucleotides, have hybridization temperatures below 41°C, explaining inefficient amplification.

EXAMPLE 5

Amplification of HIV RNA with anchor p1 primers using LNA (Locked Nucleic Acid) nucleotides in anchor sequence

In order to increase the binding efficiency of the anchor, LNA nucleotides are incorporated in the anchor. The expected Tm increase is about 3-8°C per incorporated LNA nucleotide. Four different anchor p1 primers with anchor lengths of 7nt (HIV22 LNA 1t/m4, table 1) were used in amplification. All these anchor p1 primers were tested in combination with a standard p2 primer. Amplification was performed as described in example 1. Compared to the 7nt DNA anchor primer HIV22, LNA anchor primers clearly results in an improved sensitivity of amplification (table 1) (figure 9). Compared to the 14 nt anchor primer HIV17 (table 1) or the standard p1 primer HIV1 (table 1), the performance of these LNA anchor primers is less. The position of the LNA nucleotides in the anchor sequence seems to effect amplification efficiency as a better sensitivity is obtained

using the anchor primers HIV 22 LNA 3 and 4 (table 1) compared to the primers HIV22 LNA1 and 2 (table 1).

EXAMPLE 6

Amplification of HIV RNA with anchor primers using PNA (Peptide Nucleic Acid) in anchor sequence

In order to increase the binding stability of the anchor, PNA nucleotides are incorporated in the anchor. Primers with anchor lengths of 14nt (HIV17 PNA, table 1), 12nt (HIV20 PNA, table 1), 9nt (HIV21 PNA, table 1) and 7nt (HIV22 PNA, table 1) were used in amplification in combination with a standard p2 primer (HIV2, table 1). All the anchors contain complete PNA sequences. Amplification was performed as described in example 1. Although a 10-fold decrease in sensitivity was observed as compared to DNA anchor primers, also the PNA-anchored primers were shown to be functional in NASBA (figure 10).

EXAMPLE 7

Amplification of HIV RNA with anchor p2 (reverse) primers

Amplification with anchor p2 primers (HIV27 and HIV29, table 1) was performed in combination with a standard p1 primer (HIV1, table 1). Standard p2 primers (HIV2 and HIV26, table 1) were used as a reference in combination with standard p1 primer (HIV1, table 1). Amplification was performed as described in example 1. For both amplifications, kinetics and amplification sensitivity was observed to be comparable to the reference amplification (figure 11).

EXAMPLE 8

Amplification of HIV RNA with anchor p2 primers with different anchor lengths

Amplification with anchor p2 primers with different anchor lengths varying from 22 to 14 nucleotides (HIV27, 31, 32, 33 and 29, table 1) was performed in combination with the standard p1 primer (HIV1, table 1). Amplification was performed as described in example 1. Shorten the anchor sequence of the p2-primers to 14 nucleotides results in comparable amplification efficiency as compared to the reference (HIV27 anchor primer, table 1) (figure 12).

EXAMPLE 9

Amplification of HIV RNA with anchor p1 primer and anchor p2 primer

Amplification with anchor p1 primer (HIV17, table 1) and anchor p2 primers (HIV27 and HIV29, table 1) resulted in comparable amplification efficiency compared to the standard primer set (HIV1/ HIV2, table 1) (figure 13). Amplification was performed as described in example 1.

EXAMPLE 10

Amplification of HCV RNA with anchor p1 primer

Also for the amplification of HCV RNA a NASBA was designed making use of an anchor p1 primer. Amplification with anchor p1 primer (HCV13 or HCV 49, table 2) and a normal p2 primer (HCV2, table 2) was performed. The anchor primers consist of a 14 nt anchor sequence upstream of the T7-promoter sequence and 7nt hybridizing sequence downstream of the T7-promoter sequence. Amplification with standard p1 primer (HCV1, table 2) and standard p2 primer (HCV2, table 2) was used as reference. Molecular beacon HCV-WT3 (table 2) is used as probe and in vitro obtained HCV RNA transcript as

input. Amplification was performed as described in example 1 except that the denaturing step at 65°C was performed for 5 minutes instead of 2 minutes. Although differences in sensitivity and kinetics are observed, the results show that anchored primers can be used for amplification of HCV RNA (figure 14).

EXAMPLE 11

Amplification of HCV RNA with anchor p1 primer, having a 3nt gap between the binding site of the anchor and the 3' hybridizing sequence

Amplification with anchor p1 primer (HCV22, table 2) and a normal p2 primer (HCV2, table 2) was performed. In this anchor, a 3 nt gap separates the binding site of the anchor sequence from that of the 7nt 3' hybridizing sequence. Amplification with anchor p1 primer HCV13 (table 2) and standard p2 primer (HCV2, table 2) was used as reference. Molecular beacon HCV-WT3 (table 2) is used as probe and in vitro obtained HCV RNA transcript as input. Amplification was performed as described in Example 10. The results show that a 3 nt gap can exist between the binding site of the anchor and that of the 3' hybridizing sequence (figure 15), and that these anchored primers can be used to bridge non-conserved nucleotides in the primer-binding site.

Table 1

NR°	*Sequence 5'-> 3'*
	Standard primers
HIV1 (p1)	*AATTCTAATACGACTCACTATAGGG* TGCTATGTCACTTCCCCTTGGTTCTCTCA
HIV2 (p2)	AGTGGGGGGACATCAAGCAGCCATGCAAA
HIV26 (p2)	AGTGGGGGGACATCAAGCAGC
HIV11	*AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
	Anchor p1 primers
HIV12	TGCTATGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV15	CTATGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV16	TGTCACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV17	CACTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV20	CTTCCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV21	CCCCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22	CCTTGGT *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
	Anchor p1 primers with 2'-O-Methyl nucleotides in anchor
HIV17 MET	CACTT**CCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV20 METa	CTT**CCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV20 METb	C**TTCCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV21 METa	**CCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV21 METb	**CCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 METa	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 METb	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
	Anchor p1 primers with LNA nucleotides in anchor
HIV22 LNA1	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 LNA2	CC**TTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 LNA3	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 LNA4	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
	Anchor p1 primers with PNA anchor
HIV17 PNA	**CACTTCCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV20 PNA	**CTTCCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV21PNA	**CCCCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
HIV22 PNA	**CCTTGGT** *AATTCTAATACGACTCACTATAGGG <u>AAACGGGCACGAGC</u>* TCTCTCA
	Anchor p2 primers
HIV27	AGTGGGGGGACATCAAGCAGCC *<u>GACTTCAGGACTTCAGG</u>* ATGCAAA
HIV31	GGGGGACATCAAGCAGCC *<u>GACTTCAGGACTTCAGG</u>* ATGCAAA
HIV32	GGGACATCAAGCAGCC *<u>GACTTCAGGACTTCAGG</u>* ATGCAAA
HIV33	GACATCAAGCAGCC *<u>GACTTCAGGACTTCAGG</u>* ATGCAAA
HIV29	AGTGGGGGGACATC *<u>GACTTCAGGACTTCAGG</u>* AAGCAGC
	Molecular beacon for real time detection
HIV MB WT	gcatgc ATCAATGAGGAIGCTGCAGAITGGGA gcatgc (5' FAM / 3' dabcyl labeled)

Target specific sequence in bold, T7 promoter sequence in italic, Y sequence (p1 primers) or X sequence (p2 primers) in italic and underlined, modified nucleotides in anchor with grey background

Table 2

NR°	*Sequence 5'-> 3'*
	Standard primers
HCV1 (p1)	*AATTCTAATACGACTCACTATAGGG* CAAGCACCCTATCAGGCAGTA
HCV2 (p2)	GTCTAGCCATGGCGTTAGTA
	Anchor p1 primers
HCV13	CAAGCACCCTATCA *AATTCTAATACGACTCACTATAGGG* *AAGAGGGCACGAGC* GGCAGTA
HCV22	TCGCAAGCACCCTA *AATTCTAATACGACTCACTATAGGG* *AAGAGGGCACGAGC* GGCAGTA
HCV49	CAAGCACCCTATCA *AATTCTAATACGACTCACTATAGGG* *AAACGAGCACGAGC* GGCAGTA
	Molecular beacon for real time detection
HCV WT3	gctagc ATTTGGGCGTGCCCCCGCIAGA gctagc (5' FAM / 3' dabcyl labeled)

Target specific sequence in bold, T7 promoter sequence in italic, Y sequence (p1 primers) or X sequence (p2 primers) in italic and underlined